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# Novel Purification of Vitronectin from Human Plasma by Heparin Affinity Chromatography

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**ABSTRACT.** The glycoprotein vitronectin (also called S-protein, serum spreading factor, or epibolin) promotes spreading of a variety of cultured cells, inhibits the cytotoxicity of membrane attack complex C5b-9, and modulates thrombin-antithrombin III activity. We developed a strikingly simple method to purify vitronectin from human plasma by heparin affinity chromatography. Serum was obtained from plasma by adding calcium and then centrifuging. The heparin-binding activitiy of vitronectin in human serum was activated with 8 M urea. The activated vitronectin specifically bound to heparin-Sepharose in 8 M urea and was eluted with 0.5 M NaCl containing 8 M urea. This procedure resulted in an approximately 250-fold purification of vitronectin with a 15–30% recovery; 3-6 mg of pure vitronectin were obtained from 100 ml human plasma within 2 days. The purified vitronectin preparations promoted spreading of BHK fibroblastic cells on substrates with a half-maximal activity at only 0.1 μg/ml. This new method is very simple, rapid, inexpensive, and flexible. It could probably be readily scaled up for commercial applications.

Vitronectin (also called S-protein, serum spreading factor, or epibolin) is one of two major cell-adhesive glycoproteins in mammalian plasma and serum (3, 10, 13, 20, 36); the other is fibronectin. Vitronectin is a monomeric glycoprotein detected as a mixture of 75 kilodalton (kDa) and 65 kDa polypeptides; the latter seems to be an endogenously proteolytically nicked product of the former (1, 3, 6, 13). Vitronectin binds to heparin (13), collagen (9, 16), thrombin-antithrombin III complex (15, 17, 27, 30), C5b-9 complex (25), streptococci (5, 8), and a variety of cultured cells. The cell-binding site of vitronectin involves a common cell-attachment tripeptide Arg-Gly-Asp (32, 37). Although the primary structure, cDNA sequence (18, 37), and domain structure (38) are known, published purification procedures for vitronectin are relatively cumbersome and inefficient. Human plasma and serum contain 10–40 mg of vitronectin per 100 ml (3, 13, 34), but only a few mg or less of vitronectin can be isolated with a 0.5–20% recovery efficiency through very long processes (3, 6, 28, 29, 35) or with a very expensive, commercially available monoclonal antibody (13, 38).

In pursuing studies on the biological functions of vitronectin, simpler and more

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Abbreviations used: kDa, kilodalton; SDS, sodium dodecyl sulfate; Buffer A, 5 mM EDTA, 8 M urea, and 10 mM Na-phosphate buffer (pH 7.7).

rapid methods were required for the preparation of milligram quantities of biochemically pure vitronectin. Barnes *et al.* (2) and we (11) have recently found that 8 M urea activates the heparin-binding activity of vitronectin. This led us to develop the method for purification of vitronectin by heparin affinity chromatography described in this report.

## MATERIALS AND METHODS

*Materials.* Heparin-Sepharose 4B was prepared according to Fujikawa *et al.* (7) and our final heparin-Sepharose 4B preparation contained 0.54 mg of heparin/ml hydrated gel. Fibronectin from outdated human plasma and anti-human fibronectin rabbit antibody were prepared as described previously (12, 33). Anti-human albumin antiserum was obtained from Miles-Yeda, Ltd. (Rehovot, Israel). The synthetic fibronectin peptides, Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP) and Gly-Arg-Gly-Glu-Ser-Pro (GRGESP), were a kind gift from Dr. Toshio Goto (Tansaku Res. Inst., Fujisawa Pharm., Co., Ltd., Tsukuba, Japan).

*Purification of vitronectin.* All procedures were performed at room temperature unless otherwise specified. Defrosted, outdated human plasma (100 ml) was clotted in glassware by the addition of 2 ml of 1 M $CaCl_2$ followed by incubation for 1 h at room temperature and then for 2 h at 4°C. The resulting serum was added to 0.5 ml of 0.2 M phenylmethane sulfonyl fluoride in ethanol and 2.5 ml of 0.2 M EDTA. The pH was approximately 7.7. The serum mixture was applied to a pre-column (1.5 cm in diameter) of Sepharose 4B (2 ml bed volume; 2 ml/min) and then to a heparin-Sepharose pre-column (5 ml bed volume; 2 ml/min) which had been previously washed with 5 mM EDTA and 10 mM Na-phosphate buffer (pH 7.7) containing 2 M NaCl and equilibrated with the same solution but now containing 0.13 M NaCl. The flow-through fractions were collected and added to 144 (or 96) g of urea. The total volume was adjusted to 300 (or 200) ml with distilled water to obtain a final concentration of 8 M urea. After standing for 2 h at room temperature or being boiled for 5 min, it was applied to the same heparin-Sepharose affinity column (2 ml/min) which now had been previously washed with Buffer A (5 mM EDTA, 8 M urea, 10 mM Na-phosphate buffer (pH 7.7)) containing 2 M NaCl and 10 mM 2-mercaptoethanol and then equilibrated with Buffer A. After adding the urea-treated fractions to the heparin-Sepharose column, the column was washed with 100 ml of Buffer A, then with 150 ml of Buffer A containing 0.13 M NaCl. The heparin-Sepharose column was held for 2 h after passing one column volume (5 ml) of Buffer A containing 10 mM 2-mercaptoethanol and 0.13 M NaCl through it, and then it was washed with 45 ml of the same solution to remove contaminants sensitive to disulfide reduction. Pure vitronectin was subsequently eluted from the heparin-Sepharose column with 100 ml of Buffer A containing 0.5 M NaCl. The heparin-Sepharose column was used repeatedly.

Vitronectin was also purified from human plasma as a non-heparin-binding fraction according to the procedure of Barnes and Silnutzer (3, 35) as described previously (11).

*Preparation of anti-vitronectin antibody.* Rabbit anti-human vitronectin antibody was prepared as described previously and the antibody was shown to be monospecific (1).

*Immunoassay for vitronectin, fibronectin, and serum albumin.* As described in detail previously (1), a modified sandwich enzyme-linked immunosorbent assay in which vitronectin was boiled in 2% sodium dodecyl sulfate (SDS), termed ELISA-SDS, was used to measure total quantities of vitronectin regardless of its conformation. Fibronectin was measured by an enzyme-linked immunosorbent assay as described previously (33). Immunoblotting for vitronectin was performed by the procedure of Towbin *et al.* (39) as described previously (33). The amount of contamination by human serum albumin of the

purified vitronectin preparation was estimated using the immunoblotting assay without bovine serum albumin. Instead, blocking of the nitrocellulose sheet was performed with 3% gelatin in phosphate-buffered saline and antibodies were diluted with 1% gelatin in phosphate-buffered saline.

*Cell-spreading activity.* Cell-spreading activity was measured using BHK fibroblastic cells according to the procedure of Grinnell *et al.* (10). Briefly, 96-well polystyrene tissue culture plates were coated with 50 μl of vitronectin or fibronectin at the indicated concentrations in an adhesion medium (150 mM NaCl, 1 mM $CaCl_2$, 3 mM KCl, 0.5 mM $MgCl_2$, 6 mM $Na_2HPO_4$, and 1 mM $KH_2PO_4$; final pH 7.3) for 1 h at 37°C. After discarding the coating solution, BHK cells in the adhesion medium were added and incubated at 37°C for 1.5 h. The percent of spread cells (number of spread cells per 100 attached cells) was quantitated microscopically. To test for inhibition of cell spreading by antibodies, the coated plates were treated with anti-vitronectin or anti-fibronectin antibodies at 0.1 or 0.3 mg/ml at 37°C for 1 h before addition of BHK cells. Inhibition by peptides was examined by simultaneous incubation of BHK cells with the synthetic fibronectin peptides GRGDSP or GRGESP on the vitronectin-coated plates. To test for a requirement for protein synthesis, BHK cells were pre-incubated with 20 μg/ml of cycloheximide for 10 min and then placed on the coated plates without removing the cycloheximide.

*Other procedures.* Protein concentrations of vitronectin and fibronectin were determined from absorbance measurements at 280 nm with a 1-cm path length cell using $E_{1\ mg/ml}$ values of 1.38 (6) and 1.28 (24), respectively. SDS-polyacrylamide gel electrophoresis was performed essentially according to the method of Laemmli (22).

## RESULTS

*Purification of vitronectin.* Figure 1 shows a typical elution profile for the heparin-Sepharose affinity chromatography of human serum. Vitronectin was recovered with a 15–30% efficiency by elution with Buffer A containing 0.5 M NaCl (Fig. 1, arrow c) with an approximately 250-fold increase in purification. Fibronectin contamination could not be detected and there was less than 10 ng/mg of vitronectin. The procedure produced 3–6 mg of vitronectin from 100 ml of human serum within only 2 days. No detectable proteins were eluted from the heparin-Sepharose column by a further elution with Buffer A containing 2 M NaCl. SDS-polyacrylamide gel electrophoresis indicated that vitronectin was purified as two polypeptides, one migrating at 75 kDa and the other at 65 kDa (Fig. 2, lane f), both of which were similarly stained by anti-vitronectin antibody in an immunoblotting assay (data not shown). Vitronectin once purified could be re-bound to heparin-Sepharose in 0.13 M NaCl in the absence of 8 M urea. Unexpectedly, protein components examined by SDS-polyacrylamide gel electrophoresis from the elution with 0.13 M NaCl (Fig. 1, arrow a) and the reducing agent (Fig. 1, arrow b) were indistinguishable from those of the initial flow-through fraction (Fig. 2, lanes c, d, and e). Albumin was an apparent major contaminant eluted at all steps (Fig. 2). The vitronectin preparation was contaminated with approximately 10 μg albumin/mg of vitronectin as indicated by the immunoblotting assay using anti-human serum albumin (data not shown).

*Comments on the purification procedure.* Approximately 50% of the vitronectin appeared in the flow-through fractions plus fractions eluted with 0.13 M NaCl. An additional ~7% was recovered by rechromatography of these fractions. The significant amount of vitronectin recovered by rechromatography was not obtained

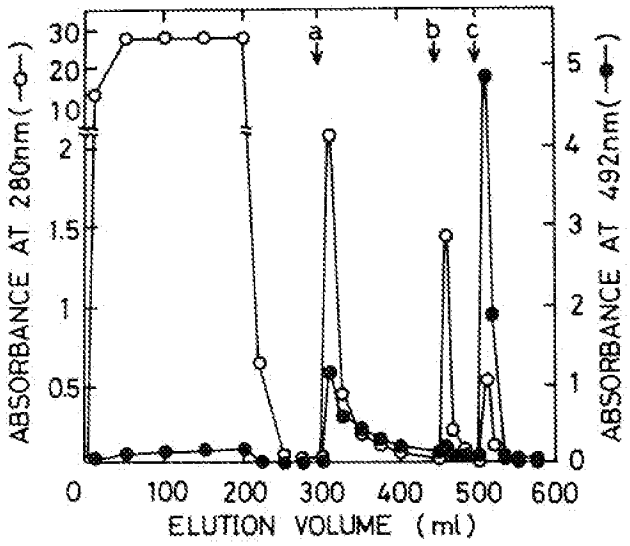


Fig. 1. Heparin-Sepharose affinity chromatography of human serum. For details, see MATERIALS AND METHODS. Briefly, 100 ml of human serum were passed through a precolumn of heparin-Sepharose, then supplemented with urea to obtain a final concentration of 8 M. The serum in 8 M urea was applied to a second heparin-Sepharose affinity column (5 ml bed volume). The column was washed with Buffer A (5 mM EDTA, 8 M urea, and 10 mM Na-phosphate buffer (pH 7.7)), then with Buffer A containing 0.13 M NaCl at arrow "a". After further washing with Buffer A containing 0.13 M NaCl and 10 mM 2-mercaptoethanol at arrow "b", vitronectin was eluted with Buffer A containing 0.5 M NaCl at arrow "c". Protein concentration (○) was determined by absorbance at 280 nm and vitronectin concentration (●) was expressed as absorbance at 492 nm as determined by a modified enzyme-linked immunosorbent assay for vitronectin (1).

from what remained as the result of initial overloading of the heparin-Sepharose column, since vitronectin appeared at a constant level throughout the flow-through fractions. Although a larger bed volume of the heparin-Sepharose slightly increased the recovery of vitronectin, vitronectin still appeared in early flow-through fractions.

Removal of fibrinogen by the addition of a final concentration of 11% (v/v) cold ethanol could be substituted for the addition of calcium. Without removal of fibrinogen by coagulation to form serum or by 11% ethanol, the plasma became very viscous and gelled in a few hours after the addition of 8 M urea, halting the flow in the heparin-Sepharose column. The gel could not be sedimented by centrifugation at 15,000 rpm for 20 min at 4°C. Fibrinogen did not contaminate the final vitronectin preparation if such viscous plasma was forced through the heparin-Sepharose column. A peristaltic pump was often needed for such viscous plasma even on a small scale. Treatment with 11% ethanol did not precipitate the vitronectin in the plasma.

The pre-column of heparin-Sepharose prior to urea treatment was used to obtain purer vitronectin. Without it, the vitronectin preparation from serum was slightly contaminated with proteins migrating at 54 kDa, 43 kDa, 39 kDa, 23.5 kDa, 20 kDa, and 18.5 kDa, and that from plasma was slightly contaminated with a protein having a molecular mass of greater than 280 kDa. The pre-column also resulted in an ~8% lower recovery of the final vitronectin preparation, because ~14% of

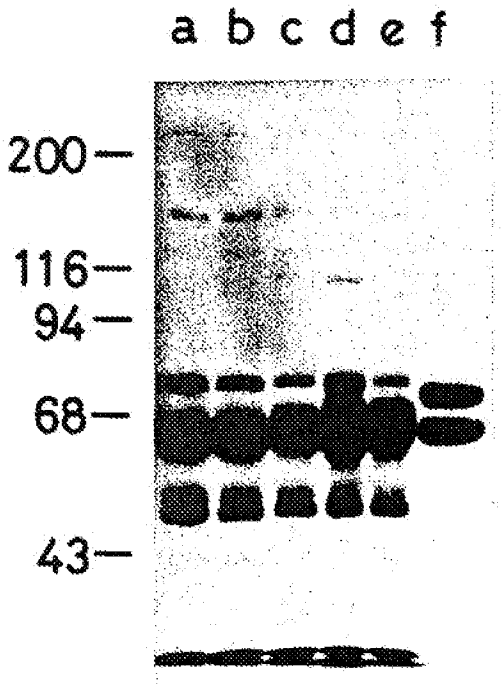


Fig. 2. SDS-polyacrylamide gel electrophoresis of the fractions from the second heparin-Sepharose affinity column. Human plasma was clotted, the resulting serum adjusted to a final concentration of 8 M urea, and this applied to a heparin-Sepharose affinity column as described in the legend to Fig. 1. The plasma, the serum obtained from the plasma, and the four protein peaks seen in Fig. 1 were analyzed by SDS-polyacrylamide gel electrophoresis after chemical reduction; (a) plasma, (b) serum, (c) flow-through fraction, (d) fraction eluted with Buffer A containing 0.13 M NaCl indicated by arrow "a" in Fig. 1, (e) fraction eluted with Buffer A containing 0.13 M NaCl and 10 mM 2-mercaptoethanol indicated by arrow "b" in Fig. 1, and (f) purified vitronectin eluted with Buffer A containing 0.5 M NaCl indicated by arrow "c" in Fig. 1. Size markers (in kDa) are shown at the left.

the vitronectin in serum and plasma was lost by its binding to the pre-column of heparin-Sepharose in the absence of 8 M urea.

The following variations resulted in substantially similar elution profiles and recovery efficiencies for vitronectin. Serum passed through the pre-column could be directly treated with Buffer A containing 0.13 M NaCl instead of with 8 M urea and distilled water. The absence of divalent cations was not essential during the procedure, because 10 mM $MgCl_2$, 1 mM $CaCl_2$, and 20 mM Tris-HCl (pH 8.0) could be substituted for 5 mM EDTA and 10 mM Na-phosphate buffer (pH 7.7) in Buffer A. Plasma and serum were ultimately diluted two or three fold before passage over the heparin-Sepharose column used for the isolation step. This dilution reduced the viscosity of plasma which increased after the addition of 8 M urea, but the dilution might not be essential for the isolation. One mM dithiothreitol could also be substituted for the 10 mM 2-mercaptoethanol. Plasma, which was fibronectin-depleted by gelatin-Sepharose affinity chromatography, produced a similar preparation of vitronectin. Serum or fibrinogen-depleted plasma in 8 M urea could be boiled for 5 min instead of standing for 2 h to save time, and this procedure did not

result in any loss of cell-spreading activity of the vitronectin preparation. Vitronectin could be eluted from the heparin-Sepharose column with Buffer A containing 0.21 M NaCl instead of 0.5 M NaCl, in which case the elution peak became broad. In the absence of 8 M urea, vitronectin could also be eluted with 0.5 M NaCl, but not with less than 0.3 M NaCl. In contrast to these changes in procedure, the following variations gave different results. When the concentration of urea was lowered to 6 M or 4 M, several proteins contaminated the vitronectin preparation. Treatment of serum with urea for less than 2 h slightly decreased the yield of vitronectin. A longer treatment of 2 days, however, did not increase the recovery of vitronectin.

*Cell-spreading activity.* Vitronectin purified by the method reported here promoted the spreading of BHK cells with a half-maximal activity at 0.1 μg/ml, which was similar to that induced by vitronectin purified by the conventional Barnes method and 10-fold higher than the specific activity induced by fibronectin (Fig. 3). However, in both preparations of vitronectin, a plateau was reached in the cell-spreading activity at around 80%, whereas with fibronectin, the activity could reach nearly 100%. Boiling for 3 min completely destroyed the cell-spreading activity induced by fibronectin, but it did not cause any decrease in the activity induced by vitronectin. Both anti-vitronectin antibody and the synthetic fibronectin peptide GRGDSP inhibited the cell-spreading activity induced by vitronectin in a dose-dependent manner, whereas a control antibody of anti-fibronectin antibody and the negative control peptide GRGESP did not inhibit cell spreading (Table 1). The effects of these peptides were in good agreement with previous reports on vitronectin's properties (14, 40). Cycloheximide at a concentration of 20 μg/ml impaired neither the activity induced by vitronectin nor that by fibronectin.

The morphology of the spread BHK cells on the vitronectin purified by the

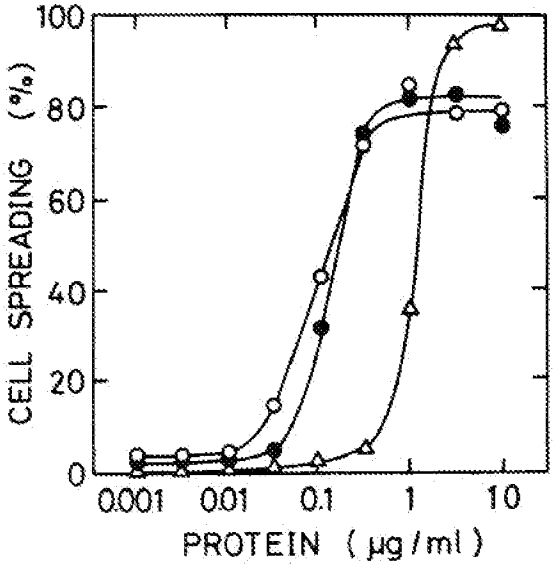


Fig. 3. Cell-spreading activity induced by vitronectin and fibronectin. Tissue culture plates were coated for 1 h at 37°C with fibronectin (△), or vitronectin purified according to the procedure reported here (○) or by the Barnes procedure (●). The number of spread BHK cells was counted after incubating the cells on the coated plates at 37°C for 1.5 h.

TABLE 1. INHIBITION OF VITRONECTIN-PROMOTED BHK CELL SPREADING BY ANTIBODIES AND SYNTHETIC FIBRONECTIN PEPTIDES

| Inhibitors | | Inhibition (%) |
|---|---|---|
| None | | 0 |
| Anti-VN | 0.1 mg/ml | 67 |
| | 0.3 mg/ml | 96 |
| Anti-FN | 0.1 mg/ml | 0 |
| | 0.3 mg/ml | 0 |
| GRGDSP | 2 μg/ml | 60 |
| | 10 μg/ml | 99 |
| GRGESP | 2 μg/ml | 2 |
| | 10 μg/ml | 3 |

Ninety six-well polystyrene tissue culture plates were coated with vitronectin (1 μg/ml) for 1 h at 37°C. To test for inhibition by antibodies, the plates were rinsed and incubated with antibodies against vitronectin (VN) or fibronectin (FN) for 1 h. After rinsing again, the plates received $2 \times 10^4$ BHK cells in 0.1 ml adhesion medium. To test for inhibition by synthetic fibronectin peptides, vitronectin-coated plates received BHK cells and synthetic fibronectin peptides simultaneously.

method reported here (Fig. 4, d) and on that purified by the Barnes procedure (Fig. 4, c) were indistinguishable. The shape of the cells on vitronectin was remarkably different from that of those on fibronectin (Fig. 4, b). On fibronectin, the BHK cells were much more extended. To examine which protein was dominant in a mixed protein assay, cell spreading was examined on plates double-coated with vitronectin and fibronectin at various concentrations. Judging from the morphologies of the spread cells, the action of fibronectin predominated at the higher protein concentrations (Table 2).

## DISCUSSION

The simple, rapid, and inexpensive purification method for vitronectin reported here provides a powerful tool for further analysis of the biochemistry and cell biology of vitronectin. This method may be scaled up for commercial purposes without any apparent problems. We have already been able to purify 45 mg of vitronectin from 2 liters human plasma within 3 days by this method. This method can also be applied to other biological fluids from any animal species, such as bovine, horse, rabbit, chicken, and porcine plasma (41).

Ruoslahti's group has been able to isolate 2–4 mg vitronectin per 100 ml human plasma by a combination of two chromatographic steps involving monoclonal anti-vitronectin antibody-Sepharose and heparin-Sepharose columns (13, 38), while Barnes' group has been able to isolate approximately 2.5 mg vitronectin per 100–150 ml human serum by a sequence of chromatography using glass beads, DEAE-cellulose, concanavalin A-Sepharose, and heparin-agarose (3, 35). The former procedure isolates a heparin-binding form, while the latter isolates a non-heparin-binding form. The very simple method reported here isolates a form similar to that from Ruoslahti's preparation and the yield is 3–6 mg vitronectin per 100 ml human plasma.

Non-heparin-binding vitronectin acquires heparin-binding activity after treatment

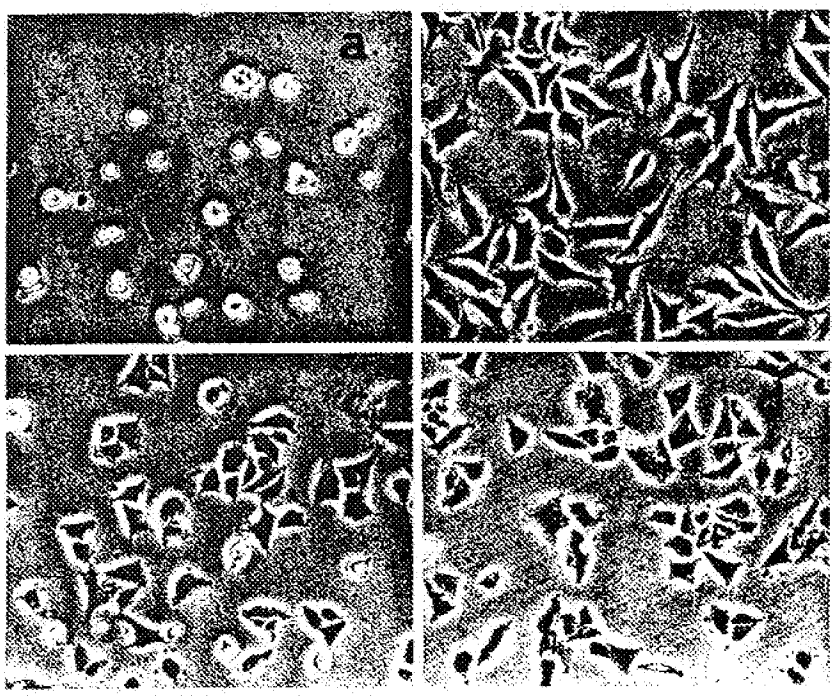


Fig. 4. Morphology of BHK cells. Spreading of BHK cells was induced as described in the legend to Fig. 3; (a) none, (b) fibronectin (10 $\mu$g/ml), (c) vitronectin purified according to the Barnes procedure (10 $\mu$g/ml), and (d) vitronectin purified by the method reported here (10 $\mu$g/ml).

with 8 M urea, 6 M guanidine-HCl, 0.05 N HCl, or boiling (2, 11). This conversion was induced during the vitronectin purification procedure reported here. Thus, vitronectin in serum also acquires heparin-binding activity after 8 M urea treatment. It is, however, unknown why approximately 2/3 of the vitronectin in human serum which passed through the pre-column was not bound to the heparin-Sepharose in the presence of 0.13 M NaCl and 8 M urea. A longer treatment of 2 days or boiling for 5 min in 8 M urea did not increase the binding efficiency. Vitronectin is known to form a complex with thrombin-antithrombin III in serum but not in plasma (15, 17, 27, 30). The purification procedure reported here produced no substantial differences between the elution profiles for, purities of, or recovery efficiencies for vitronectin from plasma and serum. Therefore, the complex formation of vitronectin with thrombin-antithrombin III causes neither serious problems in the purification procedure nor the low binding efficiency mentioned above.

We also do not understand why a number of serum proteins interacted with the heparin-Sepharose in the absence but not in the presence of the reducing agent. The interaction was not specific since a number of the serum proteins could then be eluted with the reducing agent. As vitronectin tends to form disulfide bridges itself (6), a number of serum proteins may interact with vitronectin through its reactive SH groups but not with the heparin-Sepharose.

We have recently reported that human plasma and serum can be classified into three distinct types determined by the ratio of the 75 kDa to 65 kDa vitronectin

polypeptides (21). In our preliminary experiments, purification starting with 75 kDa polypeptide-rich plasma always resulted in vitronectin preparations having equal quantities of 75 kDa and 65 kDa polypeptides (Kubota, K. and M. Hayashi; unpublished results). Thus, the vitronectin purified with the procedure described here probably does not represent the same vitronectin components found in native plasma and serum, suggesting that, at least in 8 M urea, there are heterogeneous states of vitronectin in plasma and serum. Although the reason for the heterogeneity in the biochemical states of vitronectin is unclear, it may be why only 0.5–30% of the vitronectin is recovered with a variety of purification procedures (3, 6, 28, 29, 35, this paper), even when a monoclonal antibody column, which is supposed to collect almost all vitronectin, is used (13, 38). McGuire *et al.* (23) recently reported that vitronectin purified from human plasma contains ~2.5 mol of endogenous phosphate/mol of protein. Furthermore, vitronectin can be phosphorylated at $Ser^{378}$ by an incubation in ATP. Phosphorylation may change the heparin-binding properties of vitronectin.

The effective concentration inducing BHK cell spreading was 10-fold lower for vitronectin than for fibronectin (Fig. 3). Klebe *et al.* (19) reported that fibronectin binds to a polystyrene substrate with a 68% efficiency at a concentration of approximately 6 μg/ml. In our preliminary experiments, $^{125}I$-labeled vitronectin bound to a polystyrene substrate under similar conditions with a 53–60% efficiency at concentrations ranging from 0.1 to 10 μg/ml (Izumi, M. and M. Hayashi; unpublished results). Thus, better binding of vitronectin to plastics does not appear to occur and is probably not the reason for the higher specific activity of vitronectin. The higher specific activity may rather reflect a stronger affinity of vitronectin for the vitronectin receptor than that of fibronectin for the fibronectin receptor (31).

About 20% of the BHK cells did not spread in response to the vitronectin, whereas almost all cells spread in response to the fibronectin (Fig. 3). These non-spreading cells may not express vitronectin receptors on the cell surface or may lack an intracellular pathway which depends on the activity of the vitronectin receptor.

The morphology of cells spread on vitronectin was also different from that of cells on fibronectin. Vitronectin did not induce full lateral expansion of the peripheral cytoplasm in the spread cells. This poor spreading is clearly not due to the treatment of vitronectin with 8 M urea or due to the heparin-binding activity of

TABLE 2. CHECKERBOARD ANALYSIS OF CELL-SPREADING TYPE ON DOUBLE-COATED PLATES

| VN \ FN | 10 | 3 | 1 | 0.3 | 0 |
|---|---|---|---|---|---|
| 10 | F | F | F | V | V |
| 1 | F | F | F | V | V |
| 0.3 | F | F | F | V | V |
| 0.1 | F | F | F | V | V |
| 0 | F | F | F | — | — |

Tissue culture plates were coated for 1 h at 37°C with a mixture of fibronectin (FN) and vitronectin (VN) at the indicated concentrations (μg/ml). After rinsing, BHK cells were allowed to settle on the double-coated plates at 37°C for 1.5 h. The overall morphology of the BHK cells in each well was classified by phase contrast microscopy as either a fibronectin type "F" or a vitronectin type "V" as shown in Fig. 4(b) and (d), respectively. Almost all spread cells in each well on the plate were uniformly of one type. "—" indicates almost no spreading.

the vitronectin, because Barnes' type of vitronectin had a similar effect. Knox and Griffiths (20) also reported similar morphological differences, but their vitronectin and fibronectin preparations were very crude, being separated from human serum only by sizing column chromatography. Since a mixture of fibronectin and vitronectin induced full spreading (Table 2), the poor spreading was also not the result of a tighter adhesion of cells to vitronectin-coated substrates. Nagata *et al.* (26) reported that collagen inhibits BHK cell spreading on fibronectin-coated substrates. Vitronectin, however, did not modulate it.

Boiling vitronectin for 5 min in the absence or presence of 8 M urea did not impair the spreading activity of vitronectin. This striking heat-resistance of vitronectin conflicts with a previous report by Barnes *et al.* (4). In contrast, fibronectin lost all of its cell-spreading activity after boiling.

From the differences mentioned above, the molecular mechanism of cell spreading mediated by vitronectin may involve not only different receptors (31), but also a mechanism distinct from that used by fibronectin.

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